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Heterologous boosting of nonrelated toxoid immunity during acute Puumala hantavirus infection

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ABSTRACT

Persistence of immune memory in humans is a crucial yet poorly understood aspect of immunology. Here we have studied the effect of Puumala hantavirus infection on unrelated, pre-existing immune memory by studying T cell- and antibody responses against toxoid vaccine antigens of diphtheria, tetanus and pertussis in a cohort of 45 patients. We found that tetanus- and pertussis -specific IgG concentrations elevate during acute Puumala virus infection. Increase in vaccine IgG was associated with proliferation of heterologous T cells. Interestingly, increases in tetanus-specific IgG persisted a year after the infection while pertussis-specific IgG declined rapidly; a difference in IgG kinetics resembling the difference seen after vaccination against tetanus and pertussis. These results suggest that persistence of immune memory is facilitated by heterologous boosting of old memory during memory formation against newly encountered antigens. They also show that different toxoid antigens may be treated differently. Our study gives new insight into how immune memory formation may alter pre-existing immune memory, and also shows that heterologous immunity may have an impact on vaccination outcomes.

1. Introduction

Immunological memory allows the immune system to generate rapid and effective responses to previously encountered antigens; a function necessary for gaining immunity against pathogens [1]. Classically, immunological memory was thought to be dependent on heterogeneous memory T and B cell populations, but this definition of immunological memory has recently been challenged by the discovery of non-antigen-specific memory dependent of epigenetic programming in innate immune cells [2]. Furthermore, while memory cell mediated immunological memory is in principle antigen-specific, stimulation of the immune system can amplify unrelated pre-existing memory in a phenomenon known as heterologous immunity [3,4]. This phenomenon has been identified particularly in rodents and between viral antigens [5], but its significance in humans remains unclear. Activation of heterologous bystander memory cells can be the result of T cell cross-reactivity when the antigen epitopes have close similarity [6]. Another suggested pathway for bystander activation is through production of cytokines, which would not require cross-reactivity [7]. Here we have addressed the phenomenon of heterologous immunity in patients infected by the zoonotic Puumala virus.

Puumala virus (PUUV) is a Hantavirus endemic to Europe, which is carried and spread by its reservoir host, the bank vole (Myodes glareolus) [8,9]. PUUV is the cause of a zoonotic illness known as nephropathia epidemica (NE), a hemorrhagic fever with renal syndrome [9,10]. Transmission of PUUV occurs through inhalation of aerosols containing bank vole excreta or secreta [9], but infected individuals are not infectious, except perhaps through blood contact [9,11]. Acute PUUV infection induces a strong T cell response, in which CD8+ T cells are particularly numerous [12]. Viral load in PUUV-infected patients rises after disease onset, reaching its peak on average 5 days after the occurrence of first symptoms. Viral load declines after the 7th day, leaving most

Abbreviations: PUUV, Puumala virus; NE, nephropathia epidemica; PT, pertussis toxoid; TT, tetanus toxoid; DT, diphtheria toxoid.

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patients with no detectable amounts of virus on day 10 after the onset of symptoms. Most patients are completely clear of PUUV after the 16th day after symptom debut [12], but viraemia may in cases of severe disease progression persist at least 30 days post-infection [13]. Effector T cell response to PUUV is mediated by CD8+ T cells. Proliferation of CD8+ T cells is high when the viral load peaks: over 50% of CD8+ T cells are dividing cells on day 6 after symptom debut [12].

PUUV infection has been reported to increase the number of T cells reactive against Epstein-Barr virus (EBV), a virus of the herpesviridae -family [14]. However, given the fact that EBV produces a persistent latent infection [15], it is not known, whether these PUUV-induced T cell responses to EBV depend on reappearance and new contact with EBV antigens. Memory responses against EBV could therefore be explained by reactivation of persistent EBV, and heterologous memory responses to non-persistent pathogens may not follow a similar pattern. It is therefore of considerable importance, also in terms of clinical practice and vaccinations, to understand how unrelated infections impact pre-existing immunological memory to non-persisting antigens.

2. Materials and methods

2.1. Patients and ethics

Patients presented with NE, diagnosed either by positive rapid anti-PUUV IgM test with a specificity of 99% [16] (three patients, all of whom were included in this study), or by a clinically validated serological assay at Tampere University Hospital (Tampere, Finland) between January 2005 and February 2009. Blood and urine samples were obtained at presentation (d0), and at control visits around 2 weeks (d14) and 12 months later (1y). Forty-five of the original 56 patients were available for sampling both at 2 weeks and 12 months later and were thus included. The median age of the patients included was 43 years, and the distribution was 22–73 years. Nineteen patients were female. Eight patients had an underlying medical condition (hypertension n = 5; asthma, epilepsy, rheumatic disease n = 1 each); these patients were not identifiable as outliers in our analysis. No individual data on vaccination history was available for any of the patients. Serum samples were stored in −70 °C until use. Plasma albumin and urine albumin- and creatinine concentrations were determined at the University of Massachusetts Memorial HealthCare Hospital Labs using standard clinical laboratory methods. The study was performed according to the principles of the Declaration of Helsinki. All patients gave a written, informed consent. The study was approved by the ethics committees of the Helsinki University Hospital and the Tampere University Hospital (study protocol R04180).

2.2. Analysis of antibody concentrations with ELISA

Concentrations of total IgG and IgG specific to pertussis, tetanus and diphtheria toxoids (PT, TT and DT, respectively) were measured using enzyme-linked immunosorbent assays (ELISA). Human IgG total ELISA Ready-SET-Go! kit (88–50550) by eBioscience, Anti-Bordetella pertussis toxin (PT) IgG ELISA kit (ab178634) by Abcam, Anti-Tetanus Toxoid ELISA (IgG) kit by Euroimmun (EI 2060–9601 G), and Novagnost® Diphtheria 5 S IgG kit by Siemens Healthcare Diagnostics were used for measuring total IgG, and PT-, TT-, and DT-specific IgG, respectively. For total IgG-, and TT- and DT -IgG-determinations, wells were washed using Nunc-Immuno wash 8 plate washer and in-house PBS-Tween washing buffer. For PT-IgG determinations, wells were washed manually with a pipet using the washing buffer included in the kit. The absorbances were read at 450 nm using BMG FLUOstar OPTIMA Microplate Reader (BMG Labtech).

2.3. Proliferation of vaccine-specific T cells

T cell stimulation was done as previously described [17]. Monocyte-derived dendritic cells were generated from the blood of 10 patients and used as antigen-presenting cells to activate T cells in vitro. The blood samples for the generation of antigen-presenting cells were drawn a minimum of 1 year after the acute PUUV infection. Peripheral blood mononuclear cells were isolated by Ficoll-Paque (GE Lifesciences, Buckinghamshire, UK) gradient centrifugation and cryopreserved at −134 °C with the CTL-Cryo ABC Media kit (CTL, Shaker Heights, OH, USA), according to manufacturer's instructions. Samples were immunomagnetically depleted of CD3+ cells using anti-human CD3 mAb (Immunotools, Friesoythe, Germany) and Dynabeads Pan Mouse IgG beads (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. The remaining cells were labeled with CellVue (CellVue Claret Far Red Fluorescent Cell Linker kit, Sigma-Aldrich, St. Louis, MO, USA) and enriched for monocytes by allowing adhesion to culture plates. Cells were cultured in RPMI medium with 10% human AB serum, recombinant IL-4 at 20 ng/ml (ImmunoTools) and GM-CSF at 10 ng/ml (ImmunoTools), to achieve dendritic cell differentiation. Some of the cultures were simultaneously loaded with vaccine antigens, each at 5 μg/ml: tetanus toxoid, diphtheria toxoid (both from the Finnish National Institute for Health and Welfare), and m. bovis purified protein derivative (Statens Serum Institut, Copenhagen, Denmark). The cells were activated after an overnight culture by using LPS (Sigma-Aldrich) at 1μg/ml, after which the cells were again cultured overnight. The cells were washed, and the maturation to dendritic cells was confirmed by flow cytometric analysis of expression of HLA-DR-PE, CD11c-APC (both from BD Biosciences) and CD80-FITC (ImmunoTools). $1x10^6$ stimulator cells were supplemented at 1 ml of RPMI medium with human AB-serum and recombinant IL-2 (ImmunoTools) at 25 ng/ml and mixed with $1x\ 10^6$ autologous responder cells labeled with carboxyfluorescein succinimidyl ester (CFSE) at a concentration of 1 μM (CellTrace CFSE Cell Proliferation Kit, Invitrogen), according to manufacturer's instructions. The cells were cultured for four days, after which cells were stained with CD4-APC/Cy7 (BD Biosciences, San Jose, CA, US). Flow cytometry was performed by using the Cyan instrument (Beckman Coulter, Brea, CA, USA). Proliferation was assessed by excluding the CellVue-positive stimulator cells and measuring CFSE dilution in the CD4-positive responder cells. The negative control values were subtracted from the antigen-stimulated values to obtain net proliferative values. Flow cytometry data was analyzed using the FlowJo software (TreeStar, Ashland, OR, USA).

2.4. Statistics

Statistical analysis was done using the SPSS software (SPSS Inc.). The data are shown as mean ± SD. P-values for differences between groups were calculated using the Wilcoxon signed-rank test. Correlations were calculated as Spearman's rank correlation coefficient (rho). The limit for statistical significance was 0.05.

3. Results

3.1. Heterologous T cell responses

Most vaccines are by definition administered at discrete time points and are not likely to persist for prolonged periods of time. To analyze the impact of acute PUUV infection on pre-existing T

cell memory we analyzed proliferative CD4+ T cell responses in a cohort of 10 patients at the time of presentation (d0), at d14, and at 1y after the acute phase. As antigens to stimulate the T cells we used a mixture of protein purified derivative of the Mycobacterium bovis vaccine strain (Bacillus Calmette-Guerin), tetanus and diphtheria toxoid. These antigens are present in vaccines, which are administered to effectively all Finns, and the patients were therefore expected to have underlying T cell immunity to these antigens. We did not have individualized data on the vaccination history of the patients.

At presentation and at d14 CD4 +T cells from all but one patient had a positive rate of proliferation in response to PUUV N-protein. At presentation, seven patients also showed a slight positive rate to the vaccine antigens (Fig. 1.), but the responses were generally low (mean ± SD 0.6 ± 0.7%) and significantly lower than the N-specific responses (8.4 ± 6.8%, $p < 0.01$). At d14, nine patients showed a positive response to the vaccine antigens, and in all nine it was higher than at presentation. There was also no longer a significant difference between the responses to N-protein and the vaccine antigens (data not shown). The increased vaccine responses persisted also 1 year after the acute phase in nine out of ten patients, suggesting that the cell-mediated responses to vaccine antigens were either suppressed at d0, or the acute PUUV infection boosted also unrelated responses.

3.2. Anti-pertussis and anti-tetanus IgG kinetics during acute PUUV infection and follow-up

To gain a more detailed view of the heterologous boosting of vaccine-specific memory during acute PUUV infection we analyzed humoral immunity against three structurally similar vaccine antigens: tetanus-, diphtheria- and pertussis toxoids. In Finland these antigens are usually combined in a single booster vaccine, and thus the vaccination history for all three antigens should be in broad terms similar. Of particular interest is the fact that the duration of protective immunity to the three toxoids is quite different. Immunity to tetanus and diphtheria persists for decades, whereas pertussis immunity wanes in a few years.

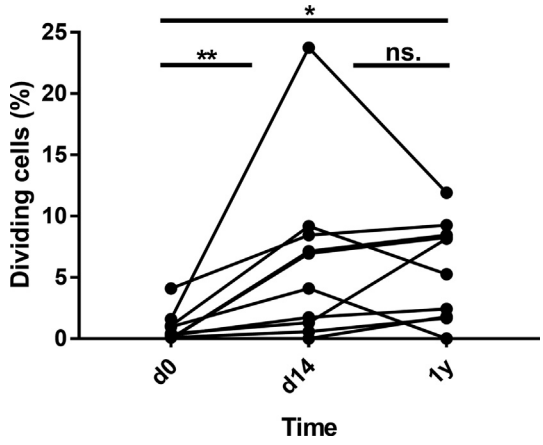

Fig. 1. Proliferation of vaccine antigen -stimulated CD4+ T cells during acute PUUV infection. The cells were stimulated with a mixture of TT, DT and M. bovis purified protein derivative and the background-subtracted net frequency of proliferating CD4+ is shown at 3 different time points: the first day of hospitalization (d0), 14 days (d14) and 1 year (1y) after hospitalization. The mean ± SD level of backgound proliferation was 2.5 ± 1.7% at d0, 3.4 ± 3.6% at d14 and 4.7 ± 3.0% at 1y. Patients are indicated by connected circles. The fraction of proliferating CD4 +T cells is shown on the y-axis as percentage. Statistical significance of difference between time points is indicated by symbols * (p < 0, 05), ** (p < 0, 01), and ns. (Not statistically significant).

To analyze the effect of acute PUUV infection on IgG antibodies specific to pertussis-, tetanus- and diphtheria toxoids (PT, TT and DT, respectively), we compared antibody levels at presentation (d0), 2 weeks after presentation (d14), and one year after presentation (1y) in a cohort of 45 patients with a serologically confirmed PUUV infection. The PUUV-specific serological status of the patients was determined at 0–4 days after presentation. TT-, DT- and PT -specific IgG antibodies were quantified from plasma samples using enzyme-linked immunoassays (ELISA). PT- and TT -specific IgG levels increased significantly between d0 and d14 (Fig. 2; $p < 0.001$ for PT, $p = 0.009$ for TT). No statistically significant change was found in the DT-specific IgG levels (Supplemental Fig. S1), and these responses were not analyzed further. There was also no significant change in the total IgG levels (Supplemental Fig. S2). Surprisingly, whereas the increased TT-specific IgG levels remained stable at the 1y time-point, the PT-specific IgG levels already showed a significant decline ($p < 0.001$).

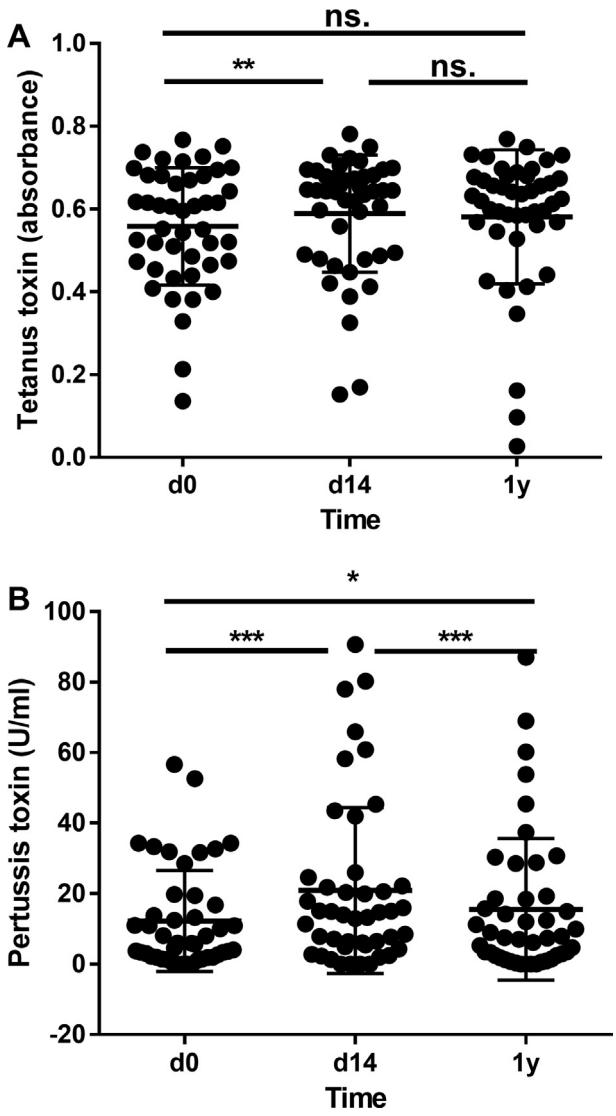

Fig. 2. Serum anti-pertussis and anti-tetanus IgG levels during acute PUUV infection and one-year follow-up. IgG levels are shown on the y-axes as absorbance for TT (A) and as concentrations for PT (B). Each patient is indicated by a circle in all time points. Means and standard deviations are shown by horizontal lines at each time point. Statistical significance of differences between time points are indicated by symbols * (p < 0, 05), ** (p < 0, 01), *** (p < 0, 001), and ns. (Not statistically significant).

Analysis of the initial antibody levels showed that at d0 all patients had detectable TT antibodies while 4 patients had no measurable PT-specific IgG. We then analyzed separately four subgroups of 10 patients: patients with either the highest or lowest initial PT and TT antibody levels. There was some overlap between the groups, since each group was determined by either PT- or TT -specific IgG levels, while disregarding the other antigen. Interestingly, the increase in PT-specific IgG during acute PUUV infection was most notable in those patients with already relatively high antibody levels (Fig. 3). This suggests that, although all patients had received multiple doses of pertussis vaccine, a persisting and measurable level of immunity was a prerequisite for heterologous boosting. In TT-specific responses the trend was less clear, and the pre-existing IgG levels did not predict the magnitude of increase.

On the average, patients with the highest initial PT-specific IgG concentrations who received detectable heterologous boosting had

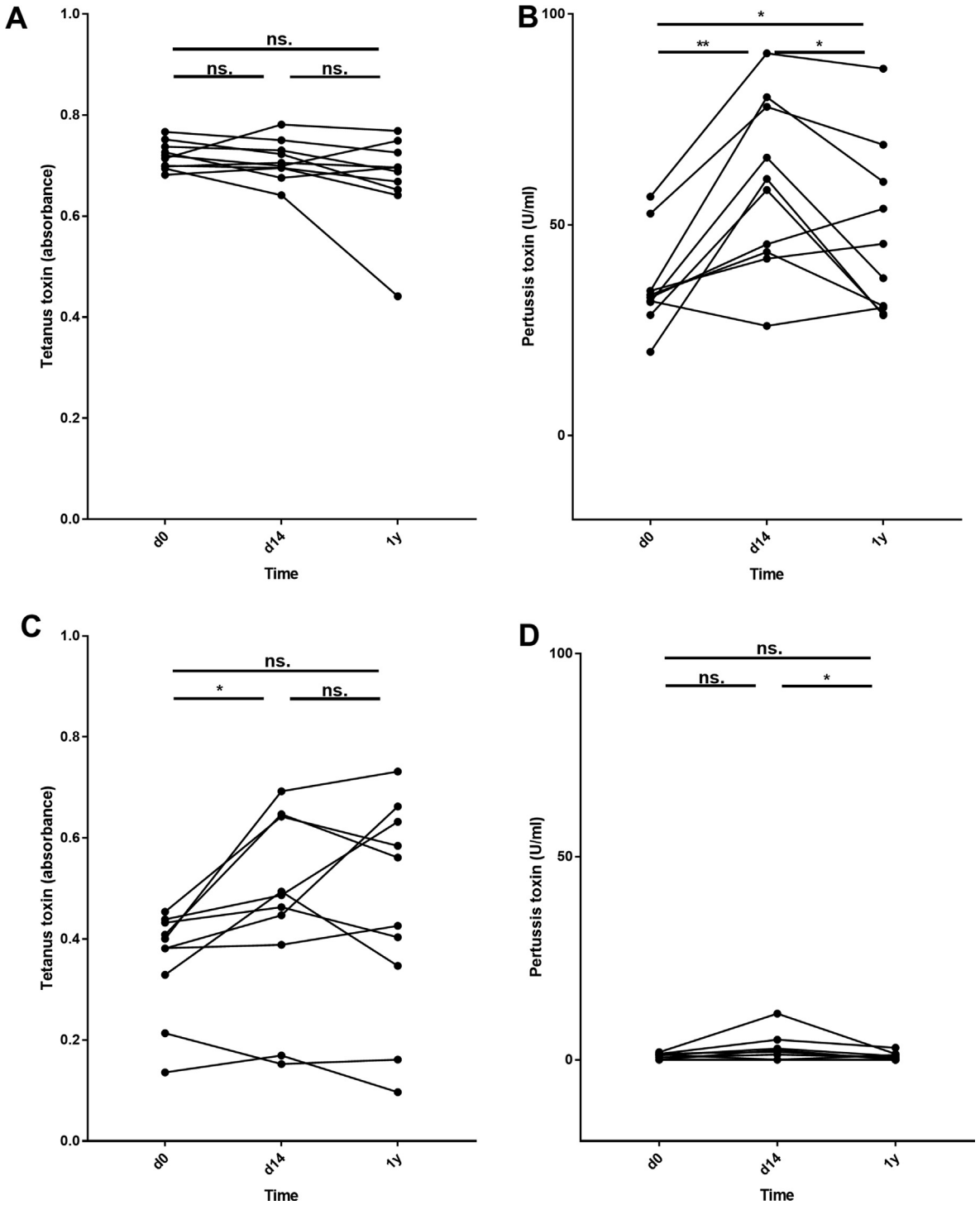

Fig. 3. Vaccine antigen -specific IgG kinetics in patients with highest and lowest baselines. Vaccine antigen -specific IgG levels of four different groups of 10 patients are shown in time-points d0, d14 and 1y. (A) and (B), respectively: TT- and PT -specific IgG levels of patients with highest initial TT- and PT -specific IgG levels. (C) and (D), respectively: TT- and PT -specific IgG levels of patients with lowest initial TT- and PT -specific IgG levels. Patients are indicated by connected circles. Statistical significance of differences between time points is indicated by symbols * ($p < 0, 05$), ** ($p < 0, 01$), and ns. (Not statistically significant).

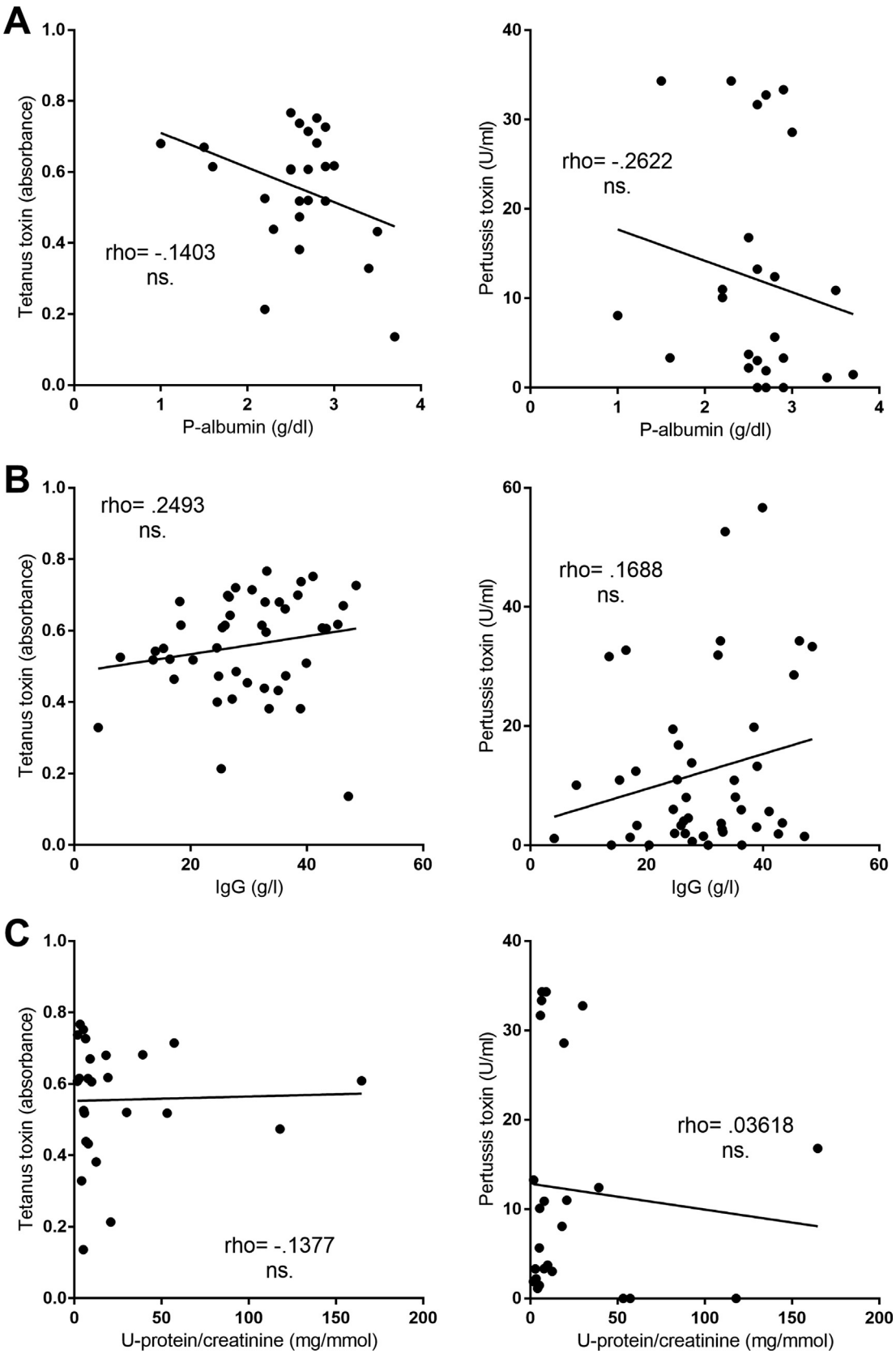

Fig. 4. Correlation of vaccine antigen -specific IgG levels with plasma albumin concentrations and urine albumin-creatinine ratios at the time of presentation. PT- and TT-specific IgG levels were correlated with plasma albumin concentrations (A), total IgG concentrations (B), and urine protein-creatinine ratios (C) measured in the acute phase. IgG levels are shown on the y axes as concentrations for pertussis toxin and total IgG, and as absorbance coefficients for tetanus toxin. Albumin concentrations, total IgG concentrations, and albumin-creatinine ratios are shown on the x-axes. Each patient is indicated by a closed circle. N = 25 for A and C, N = 45 for B. Linear regression curves, correlation coefficients (rho), and statistical significance (ns, not significant) are shown.

increased PT antibody levels also after 1 year, but in most patients the immunity already showed clear signs of waning. As expected, the patients with low initial PT antibody levels and little heterologous boosting had low PT-specific IgG levels in the 1-year control samples, as well. A similarly rapid fall was not seen in TT-specific antibodies, and on the average there was no significant difference between d14 and 1y in either the high or low initial TT-antibody group. Taken together, these findings show a difference between baseline immunity to TT and PT, but also suggest that heterologous boosting has distinct effects on the humoral responses to these two protein antigens.

We have previously shown that the single best surrogate for NE severity is the length of hospitalization [18]. To determine whether the highly variable clinical course of NE affected the heterologous responses we compared the change in TT- and PT-specific IgG levels between d0 and d14, and between d14 and 1y with hospitalization length, but found no significant correlations (Supplemental Fig. S3). No further clinical correlations were analyzed.

3.3. Excluding the effect of transient albuminuria on antibody concentrations

NE involves increased vascular permeability and acute kidney injury involving transient proteinuria [10]. Proteinuria, and also vascular leakage and acute infection itself may decrease concentrations of plasma proteins. Proteinuria in NE is non-selective, due to defects in glomerular barrier [19,20]. Low amounts of antibodies against TT and PT in acute samples in comparison with later time points could therefore be explained by excretion of protein, including antibodies, in the kidneys and blood vessels, or other mechanisms of hypoproteinemia. To exclude the possibility that the observed increase in TT- and PT-specific antibody levels was due to the recovery process and thus secondary to general loss of plasma proteins at the acute phase we compared vaccine antibody levels to plasma albumin concentrations and total IgG levels (Fig. 4 A and B, respectively). We also measured proteinuria using urine protein-creatinine ratios and compared the results with vaccine antibody levels (Fig. 4 C). Plasma albumin concentrations and urine protein-creatinine ratios were available from 25 patients in our cohort, while total IgG concentrations were determined of all included patients. However, no significant correlations were observed between toxoid antibody levels and plasma albumin, plasma IgG, or urine protein levels. These data suggest that transient albuminuria, hypoalbuminemia or hypogammaglobulinemia had no effect on vaccine antibody levels.

4. Discussion

For several reasons, PUUV infection is a promising model to study the generation and maintenance of immunological memory in humans during a natural, wild-type infection. PUUV infection results in a robust generation of CD8+ memory T-cells and PUUV-specific antibodies after the acute phase of the infection [14,21], and they have been shown to persist for at least a decade post-infection [21,22]. Primary infections are diagnosed easily through detection of PUUV-specific IgM- antibodies [23]. PUUV neither persists, nor re-infects [9], which makes it a suitable pathogen to be used in the study of immunological memory. Furthermore, PUUV infects vascular endothelium, with pathological consequences such as increased permeability, and it also affects spleen [9,24,25]. These considerations suggest that blood is likely to be a relevant tissue for analysis of effector mechanisms in these patients.

In the present study we show an increase in heterologous CD4 +T cells and TT- and PT -specific IgG concentrations in patient serum samples during acute PUUV-infection, with similar kinetics in the cellular and humoral responses. There are several caveats concerning the T cell data. First, we only analyzed proliferative responses, which may not accurately reflect other aspects of T cell function such as cytokine production. Second, since the cells were stimulated by a mixture of antigens from several vaccines, we cannot differentiate the response to individual proteins. Finally, we cannot rule out activation-induced cell death as a contributing factor to the lower frequency of responding T cells at presentation [26], so the results concerning the cellular responses must be considered preliminary.

However, no such complication pertains to the serological findings, and indeed, clinical validation of protective immunity after toxoid vaccination is based on measuring IgG levels, which adds to the relevance of the humoral data. Our data also show that vascular leakage, transient hypogammaglobulinemia or other loss of serum proteins during the infection did not account for increase in vaccine-specific antibody concentrations. Therefore, our data indicate that acute PUUV infection also activates bystander B lymphocytes specific to non-persistent antigens, enhancing measurably heterologous memory. Interestingly, whereas TT-specific IgG remained elevated during the follow-up, PT-specific IgG declined rapidly.

Since we analyzed vaccine-associated antigens, administration of these vaccines may have some effect on the data. Patient-specific data regarding vaccination status were not available for use in this study, and therefore we cannot analyze the effects of prior vaccinations on our results. However, vaccination is contraindicated during acute febrile disease. The increase in vaccine-specific immunity during the acute phase is thus highly unlikely to be caused by specific boosting. At the time of the study, the recommended interval for tetanus vaccination in Finland was 10 years [27,28], so approximately 10% of our patients would be expected to receive a booster during the 1-year follow-up. This may thus contribute to the maintenance of tetanus immunity in some patients, but overall we observed no further increase during follow-up. In contrast, pertussis vaccines are mainly administered during childhood, while in the adult version of the toxoid vaccine the acellular pertussis component is usually absent [28]. This may explain, at least in part, the generally lower baseline immunity to PT. Even so, the heterologous boosting of PT responses was roughly similar to that of TT responses.

Due to the nature of our study, we cannot address directly the mechanism responsible for the heterologous boosting. It is likely that the boosting takes place in lymphoid tissues, while we only had access to blood samples. In experimental models, heterologous immunity has been explained by antigen-specific cross-reactive mechanisms [29], or non-specific, often cytokine-mediated mechanisms [3,30], which are not mutually exclusive. In our setting, antigen-specific heterologous boosting is unlikely. Tetanus is an exceedingly rare disease in Finland [31], and natural exposure to TT is therefore highly unlikely. During the study period annual pertussis incidence in Finland ranged from 0.005% to 0.03% [31], so few if any of our patients are likely to have been infected during the follow-up. Another possible mechanism for heterologous memory responses would be T cell cross-reactivity. T cell cross-reactions to different viruses due to similarities between viral antigens have been reported, and may occur even between viruses from different families, e.g. influenza virus and hepatitis C virus [32]. However, there is no published evidence of cross-reactivity between TT, PT and PUUV antigens, and therefore cytokine-mediated boosting may be responsible.

Heterologous memory responses witnessed in this study are mainly related to long-lived plasma cells, since in the absence of reinfection they are responsible for maintaining plasma IgG levels against previously encountered pathogens. Bystander activation of

plasma cells is even less well known than that of T cells, but since surface Ig expression is low or absent in plasma cells, cytokines are likely to play a role. Most notably IL-6, APRIL (a proliferation-inducing ligand), TNF-alpha and BMCA (B-cell maturation antigen) have been associated with the maintenance of a microenvironment that promotes longevity of long-lived plasma cells [33]. Although poorly understood in humans, changes in this microenvironment are likely to take place during acute infections, and they could contribute to the augmentation of pre-existing IgG production.

Among other reasons, such as vaccine uptake, the persistent circulation of pertussis infections in most populations has been linked to the unexpectedly rapid decline in vaccine- and infection-induced immunity. Whereas vaccine-induced immunity against tetanus persists for at least 30 years [34], for reasons currently unknown pertussis vaccine efficacy declines already in 4 years [35]. Our data identify a novel factor contributing to this difference between the two pathogens: while both TT and PT-specific responses were boosted by the natural PUUV infection, only the TT responses were maintained during the follow-up. It thus seems that not only revaccinations, but also other forms of boosting have only a transient effect on the maintenance of pertussis immunity. This finding was compounded by the generally lower baseline immunity to PT, as our results showed that without measurable pre-existing PT antibodies heterologous boosting had little effect.

This study contributes to the discussion on the persistence of immune memory. CD8+ memory T cells have been reported to persist independently of antigen contact, but the impact of new memory formation has remained unclear [36,37]. Our results identify heterologous stimulation of both humoral- and T cell memory to non-persistent antigens during a natural, wild-type viral infection. They also suggest that heterologous immunity and its longevity may have measurable effects on vaccine efficacy in humans, specifically as pertains to the difference between tetanus and pertussis immunity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.02.046.

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